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What is claimed is:

1. An isolated polypeptide including an antigen binding site, the polypeptide comprising:

(a) two variable domain sequences, each variable domain sequence independently comprising at least one group of three complementarity determining regions (CDRs) interposed between framework regions (FRs), which variable domains are linked to a polypeptide linker to form a single polypeptide chain in which said framework and complementarity determining regions together define a variable region binding domain which can be immunologically reactive with an antigen, and

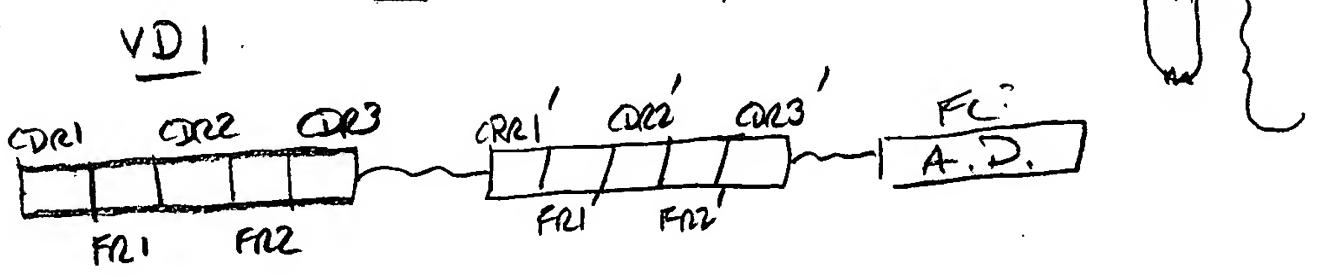
(b) an amino acid sequence that is a part of said single polypeptide chain, and has a biological activity independent of said immunological reactivity.

2. The polypeptide of claim 1, wherein said framework regions are from human immunoglobulin sequences.

3. The polypeptide of claim 1, wherein at least some of said complementarity determining regions are from human immunoglobulin sequences.

4. The polypeptide of claim 1, wherein said variable domain sequences are from human immunoglobulin sequences.

5. The polypeptide of claim 1, wherein at least some of said variable domain sequences are from human immunoglobulin sequences.




W.D. ONLY PAPER C-erbB-2 FABS

1) D.P. 5,091,513 05/21/87 5132405

6,207,804

NO ART

D.P. (ISSUES)

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WO 92/09965

SCFV

SINGLE-CHAIN FV (SFV)
BIOSYNTHETIC ANTIBODY BINDING SITES
(BABS)



C-erbB-2 FABS ONLY

complete antigen combining site, dissociation of the $V_H$ and $V_L$ domains in vivo can preclude their use as therapeutic or imaging agents. Although Moore et al., (U.S. Pat. No. 4,642,334) and Glockshuber et al., (1990, Biochem. 29, 1362-1367) disclose attempts to stabilize these Fv fragments with engineered intermolecular disulfide bonds, monovalent 50 kD Fab and Fab' fragments have, until recently, been the smallest proteins available for effective immunotargeting.

Recently, single-chain Fv (sFv) polypeptide chains of about 27 kD have been developed containing covalently linked $V_H$-$V_L$ polypeptides. The $V_H$ - and $V_L$ -domains are connected by a polypeptide linker. The resulting sFv polypeptide chains are also referred to in the art as biosynthetic antibody binding sites or BABS and preferably are encoded by a single DNA sequence. For a detailed description of these biosynthetic polypeptide chains see for example, Huston et al., 1988, Proc. Nat. Aca. Sci. USA 85: 5879-5883 or U.S. Pat. Nos. 5,091,513 and 5,132,405, all of which are hereby incorporated by reference. The sFv polypeptide chains provide attractive alternatives to intact immunoglobulins and Fab fragments due to their small size and their stability at concentrations that typically promote dissociation of natural Fv fragments. U.S. Pat. Nos. 5,091,513 and 5,132,405; Huston et al., ((1991) Methods in Enzymology 203: 46-88; Huston et al (1993) Int. Rev. Immunol. 10: 195-217) disclose the utility of sFv polypeptides, as well as single chain constructs synthesized from single DNA sequences, which may further comprise ancillary effector proteins, such as a second sFv or a cytotoxic agent.

Pack et al. ((1992) Biochem 31: 1579-1584) disclose the construction of "mini-antibodies". The mini-antibodies are sFv polypeptide chains which also include an "oligomerization domain" at their C-termini, separated from the sFv by a hinge region. The oligomerization domains comprise self-associating α-helices, for example, leucine zippers, that can be further stabilized by additional disulfide bonds. The domains are designed to be compatible with vectorial folding across a membrane, a process thought to facilitate in vivo folding of the polypeptide into a functional binding protein.

PCT application PCT/US92/09965, published Jun. 10, 1993 also discloses the construction of bivalent sFv constructs, including crosslinked dimers. However, the pharmacokinetic properties of these constructs or those disclosed by Pack et al. are not measured in vivo.

In its broadest aspects, this invention features polypeptides comprising biosynthetic antibody binding sites, DNA encoding these polypeptides prepared by recombinant DNA techniques, vectors comprising these DNAs, and methods for the production of these polypeptides.

In one aspect, the invention is based on the observation that three subregions of the variable domain of each of the heavy and light chains of native immunoglobulin molecules collectively are responsible for antigen recognition and binding. Each of these subregions, called herein "complementarity determining regions" or CDRs, consists of one of the hypervariable regions or loops and of selected amino acids or amino acid sequences disposed in the framework regions which flank that particular hypervariable region. It has now been discovered that framework regions from diverse species are effective to maintain CDRs from diverse other species in proper conformation so as to achieve true immunochemical binding properties in a biosynthetic protein. Thus, BABS produced in accordance with the invention comprise biosynthetically produced novel sequences of amino acids defining polypeptides designed to bind with a preselected antigenic material. The structure of these synthetic polypeptides is unlike that of naturally occurring antibodies, fragments thereof, or known synthetic polypeptides or "chimeric antibodies" in that the regions of the BABS responsible for specificity and affinity of binding, (analogous to native antibody variable regions) are themselves chimeric, e.g., comprise amino acid sequences homologous to portions of at least two different antibody molecules.

The invention thus provides a chimeric polypeptide defining a region capable of selective antigen binding and recognition. This chimeric polypeptide comprises amino acid sequences homologous to portions of the CDRs of the variable domain of one immunoglobulin light or heavy chain, and other sequences homologous to the framework regions, or FRs, of the variable domain of a second, different immunoglobulin light or heavy chain. Polypeptides so constructed bind a specific preselected antigen determined by the CDRs. Preferably, the chimeric polypeptides comprise an amino acid sequence homologous to at least a portion of the variable regions of a mammalian immunoglobulin, such as those of mouse, rat, or human origin. In one preferred embodiment, the biosynthetic antibody binding site comprises FRs homologous with a portion of the FRs of a human immunoglobulin and CDRs homologous with CDRs from a mouse immunoglobulin. This type of chimeric polypeptide displays the antigen binding specificity of the mouse immunoglobulin, while its human framework minimizes human immune reactions. In addition, the chimeric polypeptide may comprise other amino acid

the appropriate protease yields a complete antigen binding site or Fv fragment. This approach works well in myeloma or hybridoma expression systems.

Accordingly, it is an object of this invention to provide novel proteins comprising biosynthetic antibody binding sites including an amino acid sequence homologous to specific portions of the variable region of immunoglobulin light chain and/or heavy chain, to provide DNA sequences which encode the biosynthetic antibody binding sites, and to provide replicable expression vectors capable of expressing DNA sequences encoding the biosynthetic antibody binding sites. Another object is to provide a generalized method for producing biosynthetic antibody binding site polypeptides of any desired specificity.

In its broadest aspect, the invention features a formulation for targeting an epitope on an antigen expressed in a mammal, where the formulation contains a pharmaceutically acceptable carrier in combination with a biosynthetic construct for binding at least one preselected antigen. The dimeric construct has particular utility in diagnostic and therapeutic applications in vivo.

The invention features the synthesis and use of monomers and dimers of polypeptide constructs belonging to the class of proteins known as single-chain Fv (sFv) polypeptides. The sFv proteins described herein have superior in vivo pharmacokinetic properties, including accelerated tissue biodistribution and clearance rates relative to either intact IgG, $(Fab)_2$ dimers or Fab.

The dimeric biosynthetic construct of the invention contains two sFv polypeptide chains defined herein as follows. Each sFv polypeptide chain comprises an amino acid sequence defining at least two polypeptide domains. These domains are connected by a polypeptide linker spanning the distance between the C-terminus of one domain and the N-terminus of the other. The amino acid sequence of each domain includes complementarity determining regions (CDRs) interposed between framework regions (FRs) where the CDRs and FRs of each polypeptide chain together define a binding site immunologically reactive with a preselected antigen. Additionally, each biosynthetic binding site polypeptide chain can have an amino acid sequence peptide bonded and thus contiguous with the C-terminus of each polypeptide chain, referred to herein as a "C-terminal tail" sequence. The term "sFv'" refers hereinafter, to an sFv molecule containing such a C-terminal tail sequence. This tail sequence preferably does not contain an α-helical motif that self-associates with another polypeptide chain of similar sequence but still contains a means for covalently crosslinking two such polypeptide chains together.

In one embodiment, the biosynthetic antibody binding site is a humanized hybrid molecule which includes CDRs from the mouse 741F8 antibody interposed between FRs derived from one or more human immunoglobulin molecule. The CDRs that bind to the c-erbB-2 epitope can be found in the amino acid residue numbers 31-37, 52-68, 101-110, 159-169, 185-191 and 224-233 in SEQ ID NOS.: 1 and 2. The hybrid molecule thus contains binding sites which are highly specific for the c-erbB-2 antigen or c-erbB-2 related antigens held in proper immunochemical binding conformation by human FR amino acid sequences, which are less likely to be recognized as foreign by the human body.

The dimeric $(sFv')_2$ construct can either be homodimeric, wherein the CDR sequences on both monomers define the same binding site, or heterodimeric, wherein the CDR sequences of each sFv' monomer define a different binding site. An example of an $(sFv')_2$ heterodimer described herein having specificity for both c-erbB-2 and digoxin epitopes can be generated by combining the anti-c-erbB-2 sFv', shown in SEQ. ID. NOS.: 1 and 2 with the anti-digoxin sFv', shown in SEQ. ID. NOS.: 3 and 4. The CDRs that bind to the digoxin epitope can be derived from the anti-digoxin murine monoclonal antibody 26-10 (Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85: 5879-5883) and can be found in the amino acid residue numbers 32 through 36, 48 through 65, 101 through 107, 157 through 170, 188 through 194 and 229 through 234 in the Sequence Listing as SEQ. ID. NOS.: 3 and 4.

Radioimaging or radioimmunotherapy of tumor tissues and malignant cells are preferred aspects of this invention. Overexpression of tumor antigens such as c-erbB-2 and related cell surface antigens in malignant cells allows imaging of the malignant cell or tissue, whether it is well localized, has undergone metastasis or is exposed following cell lysis. The imaging method includes the steps of administering to a mammal a formulation comprising an sFv' or $(sFv')_2$ dimeric construct having specificity for the antigen tumor and containing a detectable moiety at a concentration sufficient to permit extracorporeal detection of the construct bound to the tumor antigen; and then detecting the biosynthetic construct bound to the tumor antigen. The formulation can be used to particular advantage in gamma scintigraphy or magnetic resonance imaging. Overexpression of c-erbB-2 or related receptors on malignant cells thus allows targeting of sFv' species to the tumor cells, whether the tumor is well-localized or metastatic. In addition, internalization of an sFv-toxin fusion protein permits specific destruction of tumor cells bearing the overexpressed c-erbB-2 or related antigen.

acceptable carrier in combination with a dimeric biosynthetic construct for binding at least one preselected antigen. The preselected antigen either may be an antigen expressed on the surface of a cell or an intracellular component exposed upon lysis of the cell. The sFv, sFv' and $(sFv')_2$ constructs disclosed herein have particular utility as <u>in vivo</u> targeting agents for detecting malignant cells in a mammal. In a particularly useful embodiment, the constructs disclosed can be used to target the c-erbB-2 or c-erbB-2-related antigens which are overexpressed in certain breast and ovarian cancers. In another embodiment, radioimmunotargeting using radiolabeled $(sFv')_2$ constructs will be useful for therapeutic as well as diagnostic applications.

Provided below are detailed descriptions of biosynthetic sFv, sFv' and $(sFv')_2$ dimers, useful in the compositions and methods of the invention, together with methods for their construction and administration. Also provided are numerous, non-limiting examples which demonstrate the suitability of these constructs as <u>in vivo</u> targeting reagents for diagnostic and therapeutic applications. More specifically, the examples demonstrate: the construction and expression of sFv polypeptides (Example 1); the renaturation, dimerization and purification of sFv' proteins (Example 2); and the immunoreactivity of the monomeric and dimeric sFv proteins (Example 3).

<u>Construction of Biosynthetic Single Chain Fv Proteins.</u>

Each of the sFv and sFv' proteins have amino acid sequences that define at least two polypeptide domains. The polypeptide domains are connected by a polypeptide linker spanning the distance between the C-terminus of one domain and the N-terminus of the other. The amino acid sequence of each domain includes complementarity determining regions (CDRs) interposed between framework regions (FRs), where the CDRs and FRs of each polypeptide chain together define a binding site immunologically reactive with a preselected antigen. Preferable polypeptide linkers can be readily tested by one of skill in the art to select a linker of optimal length and amino acid composition.

In the case of the sFv' proteins, each polypeptide chain has an additional C-terminal tail amino acid sequence having a substantially non-self-associating structure. More specifically, this is a sequence that does not interact appreciably with a similar sequence under physiological conditions, as is the case for example with the α-helical leucine zipper motifs found in DNA binding proteins. Each tail sequence also contains a means for crosslinking two such sFv' polypeptide chains together to form an $(sFv')_2$ dimer.